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P-selectin induces the expression of tissue factor on monocytes

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ABSTRACT **P-selectin on activated platelets and stimulated endothelial cells mediates cell adhesion with monocytes and neutrophils. Since activated platelets induce tissue factor on mononuclear leukocytes, we examined the effect of P-selectin on the expression of tissue factor activity in monocytes. Purified P-selectin stimulated tissue factor expression on mononuclear leukocytes in a dose-dependent manner. Chinese hamster ovary (CHO) cells expressing P-selectin stimulated tissue factor procoagulant activity in purified monocytes, whereas untransfected CHO cells and CHO cells expressing E-selectin did not. Anti-P-selectin antibodies inhibited the effects of purified P-selectin and CHO cells expressing P-selectin on monocytes. Incubation of CHO cells expressing P-selectin with monocytes leads to the development of tissue factor mRNA in monocytes and to the expression of tissue factor antigen on the monocyte surface. These results indicate that P-selectin upregulates the expression of tissue factor on monocytes as well as mediates the binding of platelets and endothelial cells with monocytes and neutrophils. The binding of P-selectin to monocytes in the area of vascular injury may be a component of a mechanism that initiates thrombosis.**

Blood clotting is a host defense mechanism that, in parallel with the inflammatory and repair responses, preserves the integrity of the vascular system after tissue injury (1). Platelets, leukocytes, and endothelial cells are among the cellular components critical for this process. The plasma blood clotting proteins participate in a molecular cascade in which tissue injury activates blood coagulation, leading to the formation of a fibrin clot (2). The response to vascular injury culminates in the formation of a platelet plug, the deposition of leukocytes in injured tissue, and the initiation of inflammation and wound healing. Blood coagulation is initiated through the action of tissue factor. Normally not exposed to blood, tissue factor is an integral membrane protein expressed constitutively on the surface of nonvascular cells. Monocytes and endothelial cells can be induced to express tissue factor on their surface (3, 4). Monocytes can be activated by endotoxin (5), immune complexes (6), certain cytokines (7), and platelets (8–11), leading to tissue factor expression, whereas endothelial cells express tissue factor when stimulated by certain cytokines (12). Tissue factor, with a molecular weight of 43,000 (13, 14), binds factor VII and factor VIIa to form the tissue factor/factor VIIa complex that activates factor IX and factor X (15).

P-selectin is a cell adhesion molecule that mediates the interaction of platelets and endothelial cells with neutrophils and monocytes (16, 17). P-selectin, a member of the selectin family of adhesion molecules (18–21), is an integral membrane protein found in the α granules of platelets (22, 23) and the Weibel–Palade bodies of endothelial cells (24, 25). Upon stimulation of these cells by agonists such as thrombin, P-selectin is phosphorylated (26, 27) and rapidly translocated to the plasma membrane (23). P-selectin is a lectin that binds to lineage-specific carbohydrates on the surface of monocytes and neutrophils (28–30). This protein binds to a mucin-like glycoprotein PSGL-1 that must be properly glycosylated to retain functional properties as the P-selectin ligand (31). P-selectin on platelets mediates the accumulation of leukocytes into the growing thrombus during experimental thrombosis *in vivo* (32). Inhibitory antibodies that block the interaction of P-selectin on platelets with the P-selectin ligand on leukocytes inhibit the uptake of leukocytes into the thrombus and inhibit the magnitude of thrombus formation. These experiments have demonstrated that P-selectin mediates monocyte and neutrophil interaction with activated platelets *in vitro* and *in vivo*. The potential exists for stimulation of leukocyte effector function by P-selectin binding. To evaluate this potential, we have examined the ability of P-selectin to upregulate tissue factor expression on monocytes. In the current study, we demonstrate that P-selectin induces the expression of tissue factor on monocytes exposed to P-selectin.

EXPERIMENTAL PROCEDURES

Proteins and Cell Lines. P-selectin was purified from platelets by detergent extraction of platelet membranes, heparin-Sepharose chromatography, and affinity chromatography using AC1.2 (30). P-selectin appeared homogeneous by SDS gel electrophoresis. Production of the noninhibitory anti-P-selectin antibody AC1.2 and the inhibitory P-selectin antibody GA6 have been described (16, 32). The inhibitory anti-tissue factor antibody HTF1 (33) was the gift of Yale Nemerson (Mt. Sinai School of Medicine). Chinese hamster ovary (CHO) cells expressing P-selectin (CHO:P-selectin) or E-selectin (CHO:E-selectin) adhere to leukocytes, in contrast to CHO cells (30).

Cell Isolation and Culture. Blood was obtained from normal volunteers and anticoagulated with 0.1 vol of 3.8% sodium citrate/0.15 M NaCl. Erythrocytes and leukocytes were sedimented at 150 × *g* for 15 min at 10°C. Platelet-rich plasma was used for platelet isolation (11). The sedimented erythrocytes and leukocytes were adjusted to the original volume with 0.38% sodium citrate/0.15 M NaCl, sedimented, and layered onto Ficoll/Hypaque (Pharmacia). After centrifugation at 400 × *g* for 30 min at 8°C, mononuclear cells were removed, diluted in 0.38% sodium citrate/0.15 M NaCl, and sedimented at 450 × *g* for 7 min at 8°C. The pellet was resuspended in buffer and the cells were washed three times. Mononuclear cells were resuspended in RPMI 1640 medium

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Abbreviations: GAPDH, glyceraldehyde phosphate dehydrogenase; LPS, lipopolysaccharide.

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supplemented with penicillin (100 units/ml) and streptomycin (100 μg/ml) at a cell concentration of 10^7 cells per ml. Cell viability (>94%) was assessed by trypan blue exclusion. Platelet contamination was 0.3–0.5 platelet per mononuclear cell. The monocytes in this population, as assessed by nonspecific esterase staining, were 25–30%. Monocytes were purified by using a discontinuous Percoll density gradient. Mononuclear cells resuspended in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum were layered onto a 46% isosmotic Percoll solution. After centrifugation at 4°C for 30 min at 550 × *g*, the cells at the RPMI 1640 medium/Percoll interface were collected and washed twice at 440 × *g* in serum-free RPMI 1640 medium. These preparations contained about 85% monocytes and 15% lymphocytes. Mononuclear cells were incubated with platelets, purified P-selectin, or CHO cells at 37°C in sterile, pyrogen-free stoppered test tubes or in tissue culture plates under 5% CO_2/95% air.

Control of Endotoxin Contamination. All reagents used for cell isolation and culture were prepared with endotoxin-free water. Solutions were prepared in glassware rendered endotoxin-free by high temperature. All reagents showed a level of endotoxin contamination, as assessed by a chromogenic *Limulus* assay (BioWhittaker), lower than 0.1 endotoxin unit/ml. All experiments were performed under sterile conditions.

Coagulant Activity Assay. Except where otherwise indicated, cells were disrupted by three freeze–thaw cycles prior to measurement of procoagulant activity by a one-stage clotting assay (11). In some experiments, intact cells were used. Intact or disrupted cells (100 μl) were mixed with 100 μl of normal human plasma at 37°C. After 30 sec, 100 μl of 25 mM $CaCl_2$ at 37°C was added to the mixture and the time to clot formation was recorded. The values were converted to arbitrary units of procoagulant activity by comparison with a standard curve obtained by using a human brain thromboplastin standard (U.K. 1) from the reference laboratory (gift of L. Poller, Manchester, U.K.). This preparation was assigned a value of 1000 units for a clotting time of 20 sec. All experimental results shown are the averages of duplicate or triplicate independent cultures within one experiment.

Quantitative PCR. Oligonucleotide primers corresponding to bp 178–198 (sense) and bp 495–515 (antisense) of the human tissue factor coding sequence and bp 64–86 (sense) and bp 581–603 (antisense) in the coding sequence of human glyceraldehyde phosphate dehydrogenase (GAPDH) were synthesized. Quantitative PCR was performed with 7 μl of cDNA from 100 μl of total cDNA resulting from reverse transcription of 1 μg of RNA. The assay mixture contained 10 mM Tris·HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.4 μg of each primer, 250 μM each dNTP, 2.5 units of *Taq* polymerase. The amplification conditions were 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min. After 25 cycles, the PCR products from GAPDH mRNA (528 bp) and tissue factor mRNA (317 bp) were analyzed by electrophoresis in a 2% agarose gel stained with ethidium bromide. For tissue factor, Southern blot analysis was performed on 8 μl of the PCR product using the labeled 317-bp fragment as a probe. The relative intensity of the bands visualized by autoradiography was measured by laser densitometry (34).

Fluorescence Microscopy. Mononuclear cells were grown on glass slides in the presence of buffer, lipopolysaccharide (LPS) (*Escherichia coli* 055:B5W; Difco), or P-selectin for 6 hr. The cells were fixed with 2% paraformaldehyde for 1 hr at 4°C and the reaction was quenched with TBS/10 mM NH_4Cl for 10 min. After incubation with 2% bovine serum albumin/phosphate-buffered saline (BSA/PBS) for 30 min and 2% BSA/PBS/0.2% human immunoglobulin for 30 min, the cells were incubated with HTF1 (5 μg/ml in 2% BSA/PBS/0.2% human immunoglobulin for 30 min at 23°C. After washing, fluorescein isothiocyanate-conjugated anti-mouse immunoglobulin was reacted with the cells for 30 min at 23°C. The cells were examined with a Zeiss fluorescence microscope.

RESULTS

The incubation of platelets with mononuclear leukocytes leads to expression of procoagulant activity. Mononuclear cells or platelets alone incubated for 16 hr at 37°C expressed minimal procoagulant activity. However, when platelets and mononuclear cells were coincubated under these conditions, we observed a 50- to 100-fold increase in procoagulant activity, confirming earlier results (8). This procoagulant activity is due to tissue factor inasmuch as stimulated mononuclear cells incubated with the inhibitory anti-tissue factor antibody HTF1 (30 μg/ml) expressed no procoagulant activity.

Purified P-selectin induced tissue factor expression by 50- to 100-fold under the conditions used (Fig. 1*A*). Tissue factor expression was inhibited if GA6, an inhibitory monoclonal P-selectin antibody, was added to cells prior to P-selectin exposure. This P-selectin preparation did not have detectable endotoxin, as monitored in the *Limulus* assay. However, endotoxin is a well-known potent stimulator of tissue factor expression in monocytes (5, 35). To distinguish between upregulation of tissue factor due to P-selectin and that due to contaminating endotoxin that may be present below the levels of detection of the *Limulus* assay, P-selectin was heated at 100°C for 30 min to denature the protein. Heat denaturation of P-selectin abolished the upregulation of tissue factor activity. In contrast, LPS, in its native form and after heating at 100°C for 30 min, stimulated tissue factor activity. These results indicate that P-selectin, and not minor endotoxin contaminants, upregulates tissue factor activity on monocytes. Intact monocytes stimulated by either P-selectin or endotoxin expressed ≈15% of the tissue factor activity of lysed cells. This is similar to the results of Drake *et al.* (36) and Levy *et al.* (37), where the tissue factor activity of intact cells was 18–21% that of lysed cells. These results indicate that the tissue factor antigen expressed on the cell surface after P-selectin exposure (see below) is functional.

The expression of tissue factor activity as a function of P-selectin concentration was studied. Expression of tissue factor activity is dependent on P-selectin concentration; the response was saturable (Fig. 1*B*). This activity was inhibited with an inhibitory anti-tissue factor antibody.

Unstimulated monocytes do not contain tissue factor in a storage pool nor is any tissue factor expressed on the cell surface (36). After stimulation, the kinetics of tissue factor expression in monocytes induced by P-selectin was compared to that induced by LPS. Tissue factor expression induced by P-selectin was observed at 4 hr, peaked at 6 hr, and then gradually decayed (Fig. 1*C*). The peak of P-selectin-induced tissue factor preceded the LPS-induced peak of tissue factor. The low level of tissue factor activity measured in the absence of P-selectin or LPS represents a low level of spontaneous activation of monocytes in culture. Although the endotoxin level in the tissue culture medium is below the sensitivity limit of the amebocyte lysate assay, we cannot eliminate a small amount of endotoxin as the cause of monocyte activation.

Cells expressing P-selectin also mediate the induction of tissue factor expression; we compared CHO:P-selectin with untransfected CHO cells for their ability to induce tissue factor expression on monocytes. CHO cells incubated with mononuclear cells had no effect on the development of tissue factor activity (Fig. 2*A*). However, CHO:P-selectin cells incubated with mononuclear cells yielded approximately a 10-fold increase in tissue factor activity. This activation was

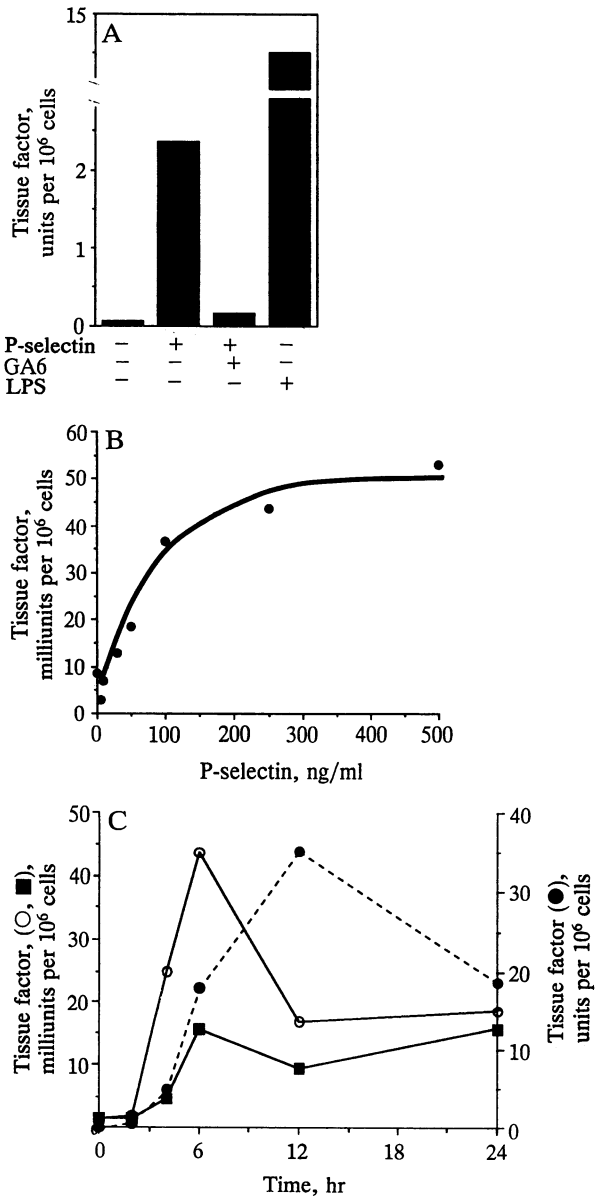

FIG. 1. Effect of purified P-selectin on tissue factor expression in monocytes. (*A*) Tissue factor expression was measured in monocytes that were untreated or exposed to P-selectin (250 ng/ml), P-selectin plus GA6 (100 μg/ml), or LPS (100 ng/ml) for 6 hr at 37°C. (*B*) Effect of increasing concentrations of purified P-selectin on tissue factor expression. Mononuclear cells were incubated with P-selectin at various concentrations in stoppered, endotoxin-free tubes at 37°C. After 6 hr, cells were assayed for tissue factor activity. (*C*) Kinetics describing the development of tissue factor expression in mononuclear cells as induced by P-selectin. Mononuclear cells were incubated with buffer, P-selectin (250 ng/ml), or LPS (100 ng/ml). After the indicated time, the cells were assayed for tissue factor activity at various time intervals. ○, P-selectin; ●, LPS; ■, buffer control.

blocked in the presence of the anti-P-selectin antibody GA6. While both isolated P-selectin and CHO:P-selectin cells induce tissue factor in monocytes, activated platelets elicit significantly higher levels of tissue factor when incubated with monocytes.

Although among mononuclear leukocytes the monocyte is the only cell capable of expressing tissue factor (38), we confirmed that the cell responsible for P-selectin-induced upregulation of tissue factor expression was the monocyte. Monocytes isolated from mononuclear cells with a Percoll density gradient, when incubated with CHO:P-selectin, also

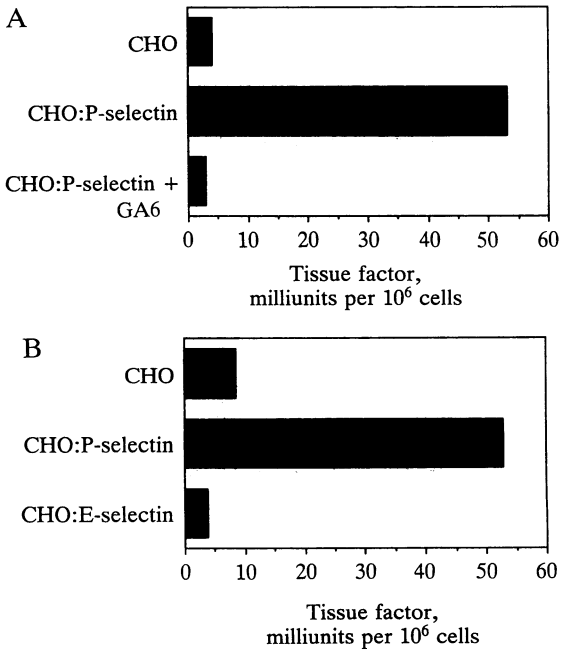

FIG. 2. CHO:P-selectin induces expression of tissue factor in monocytes. (*A*) CHO cells, either naive (CHO) or CHO:P-selectin, were cultured to confluency. Mononuclear cells were added and incubated for 6 hr in the presence or absence of $F(ab')_2$ fragments of GA6 (100 μg/ml) before assay for tissue factor activity. (*B*) CHO, CHO:P-selectin, or CHO:E-selectin cells were cultured as described above. Percoll gradient-purified monocytes were incubated as described above before assay for tissue factor activity.

expressed tissue factor activity (Fig. 2*B*). We asked whether the monocyte stimulation induced by CHO:P-selectin was due to P-selectin or whether the binding of monocytes to CHO cells mediated by any cell adhesion molecule might elicit adhesion-dependent cell activation. Monocytes were incubated with CHO:E-selectin cells (30). Although monocytes express an E-selectin ligand, which is distinct from the P-selectin ligand (30, 31, 39), monocytes bound to CHO:E-selectin cells did not express tissue factor (Fig. 2*B*).

To determine the steady-state levels of tissue factor mRNA, we performed quantitative reverse transcriptase PCR (40, 41). RNA prepared from untreated control cells, cells treated with LPS, P-selectin, or P-selectin plus the GA6 antibody was reverse transcribed and used for parallel assay of tissue factor mRNA and GAPDH PCR amplification. The expected PCR product for tissue factor (317 bp) was obtained and its identity was confirmed by restriction analysis with *Acc* I and *Rsa* I. To quantify the effect of P-selectin on tissue factor mRNA levels, we performed a Southern blot analysis of the PCR products during amplification. The PCR product from the control cells is barely visible, indicating that the expression of tissue factor mRNA is negligible (Fig. 3). The level of tissue factor mRNA was increased by ≈50-fold in cells exposed to LPS or to P-selectin. GA6 suppressed the level of tissue factor mRNA. Control experiments measuring the levels of GAPDH mRNA indicated similar mRNA levels in control and P-selectin- and LPS-treated cells, indicating that the efficiency of reverse transcription was comparable among the experimental groups. For both tissue factor and GAPDH, an identical PCR performed in parallel without including reverse transcriptase gave no amplification products, thus ruling out PCR carryover.

To confirm that P-selectin upregulates tissue factor expression on the surface of monocytes, mononuclear cells were cultured on glass slides and incubated for 6 hr with P-selectin, LPS, or buffer. The adherent monocytes incu-

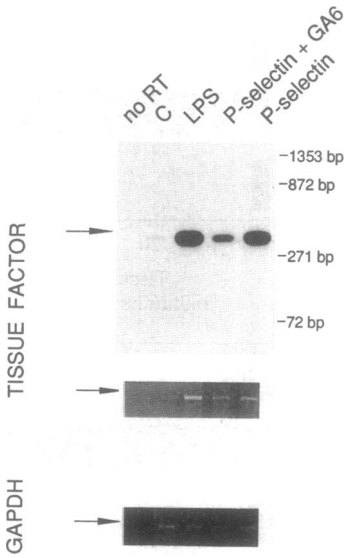

FIG. 3. Effect of P-selectin stimulation on tissue factor mRNA levels. Reverse transcriptase PCR analysis of tissue factor and GAPDH mRNA expression in monocytes untreated (C) or exposed to LPS (100 ng/ml), P-selectin (250 ng/ml) plus GA6 (100 μg/ml), or P-selectin alone for 6 hr at 37°C. (*Top*) Analysis of tissue factor mRNA by Southern blotting. Tissue factor cDNA (317 bp) is indicated by the arrow and the position of the molecular weight markers is indicated. (*Middle*) Ethidium bromide staining of the same PCR products. The specific tissue factor cDNA product (317 bp) is indicated by the arrow. (*Bottom*) Ethidium bromide staining of reaction products. The GAPDH cDNA product (528 bp) is indicated by the arrow. A parallel reaction without reverse transcriptase was carried out to test possible contamination (no RT).

bated with P-selectin or LPS were reactive with an anti-tissue factor antibody, while control monocytes incubated with buffer were not (Fig. 4).

DISCUSSION

The initiation of blood coagulation following tissue injury requires the exposure of tissue factor on the cell surface to plasma factor VIIa, a vitamin K-dependent clotting enzyme that circulates at low concentration in the blood (42). The tissue factor/factor VIIa complex on the cell surface activates both factor IX and factor X, thus leading to the sequential conversion of the blood clotting proenzymes to enzymes and the generation of a fibrin clot. Tissue factor plays a pivotal role in this process insofar as the expression of tissue factor activity is required for initiation and propagation of the signals necessary for clot formation. Nonvascular cells express tissue factor constitutively. During tissue injury, these cells make contact with flowing blood, thus initiating blood clotting on a rapid time scale. In contrast, monocytes and endothelial cells do not express tissue factor constitutively but only after cell stimulation (12, 43, 44). *De novo* synthesis of tissue factor by cells in culture requires at least 2 hr before tissue factor activity is expressed on the cell surface (43).

P-selectin is an adhesion molecule on activated platelets and stimulated endothelial cells that mediates the binding of certain leukocytes to these cells. The P-selectin ligand expressed on these cells include the Le^x carbohydrate structure, sialic acid, and a protein component PSGL-1 (31). The binding of P-selectin to leukocytes may also up- or down-regulate cell functions. Since monocytes express the P-selectin ligand and synthesize tissue factor after cell stimulation, we have evaluated whether P-selectin binding can lead to the expression of tissue factor on monocytes.

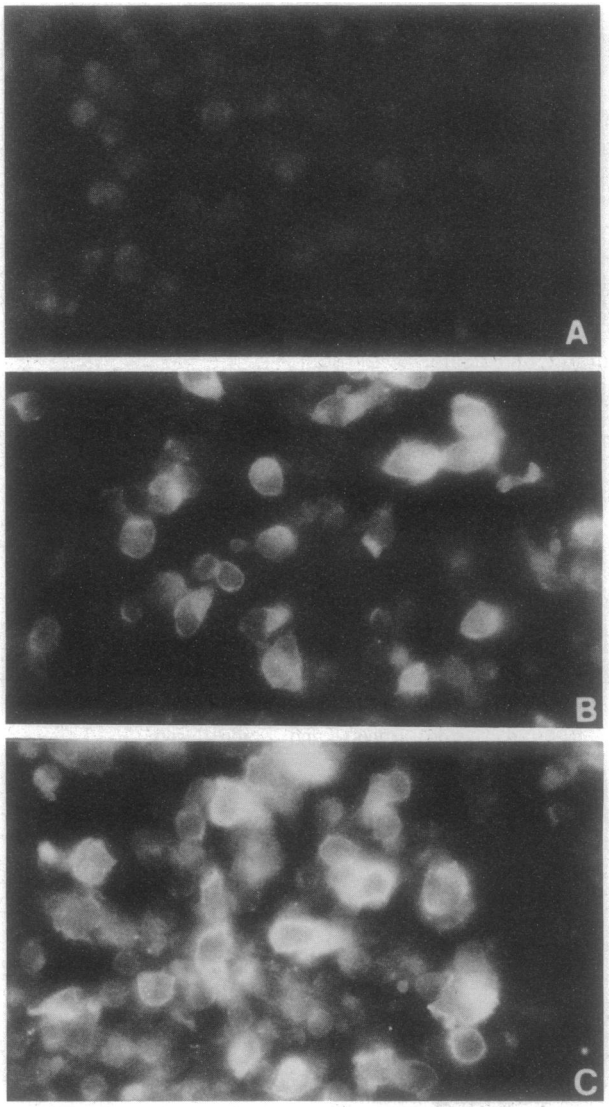

FIG. 4. Immunofluorescence staining of P-selectin-stimulated monocytes with a tissue factor antibody. Mononuclear cells were cultured on glass slides and stimulated with P-selectin, LPS, or buffer control for 6 hr. The cells were stained with fluorescein isothiocyanate-conjugated HTF1, an anti-tissue factor antibody. (*A*) Buffer control. (*B*) P-selectin. (*C*) LPS.

We have demonstrated that P-selectin in three distinct forms is capable of upregulating tissue factor: (*i*) platelets, which express P-selectin on the cell surface upon activation; (*ii*) CHO cells stably transfected with the P-selectin; (*iii*) purified P-selectin. Interaction of platelets or CHO:P-selectin with monocytes through P-selectin appears sufficient to initiate signal transduction for tissue factor expression since inhibitory antibodies to P-selectin block cell–cell interaction and the tissue factor response. However, activated platelets induce significantly more tissue factor expression than do either P-selectin or CHO:P-selectin cells. While P-selectin plays an important role in tethering neutrophils to activated endothelial cells, activation of the neutrophils, as measured by increases in cytosolic Ca^{2+}, upregulation of CD11/CD18, change in morphology, or priming for granule secretion, requires participation of a second molecule, platelet-activation factor, on the surface of activated endothelial cells (45). It is likely that in addition to P-selectin the activated platelet provides a second ligand either soluble or on the surface of the platelet that augments the synthesis of tissue factor by bound monocytes.

The potency of P-selectin alone in upregulating the synthesis of tissue factor by monocytes is small compared to LPS. However, the relative low potency of P-selectin as measured in our experiments does not exclude a physiologic role for P-selectin in thrombogenesis. For example, we have recently demonstrated in an *ex vivo* model that fibrin deposition after leukocyte accumulation on a vascular graft is inhibited by anti-P-selectin antibodies (32). Low levels of tissue factor expression as mediated by P-selectin may be significant *in vivo* in fibrin generation during vessel wall injury. Alternatively, P-selectin may be just one component required for the initiation of tissue factor expression; for example, a second molecule (e.g., 12-HETE) significantly amplifies the signal for tissue factor expression (unpublished results).

Blood coagulation is a host defense mechanism that involves an immediate, rapid response to the loss of integrity of a closed vascular system and a slower, delayed response that leads to thrombogenesis, inflammation, and wound healing. Tissue factor on nonvascular cells, which becomes accessible to flowing blood during vessel injury, is responsible for the immediate response. In contrast, the delayed expression of tissue factor in the region of tissue injury may be critical to maintenance of thrombus. In our proposed model, neutrophils and monocytes accumulate in the area of tissue injury due to the expression of P-selectin on activated platelets that collect in the subendothelium and on stimulated endothelium in the injured vasculature. Upon interaction with P-selectin, and possibly other required ligands, on platelets and endothelial cells, monocytes upregulate the *de novo* synthesis of tissue factor. Within several hours, tissue factor expression on the cell surface supports and maintains the local activation of blood coagulation and the deposition of fibrin. It is likely that P-selectin initiates a general cell response, of which tissue factor biosynthesis is but one of a series of metabolic and synthetic pathways activated. P-selectin-mediated effector function in leukocytes will likely play an important role in inflammation and thrombosis.

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